

# Rak Functions as a Tumor Suppressor by Regulating PTEN Protein Stability and Function

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## SUMMARY

Expression of the PTEN tumor suppressor is frequently lost in breast cancer in the absence of mutation or promoter methylation through as yet undetermined mechanisms. In this study, we demonstrate that the Rak tyrosine kinase physically interacts with PTEN and phosphorylates PTEN on Tyr336. Knockdown of Rak enhanced the binding of PTEN to its E3 ligase NEDD4-1 and promoted PTEN polyubiquitination, leading to PTEN protein degradation. Notably, ectopic expression of Rak effectively suppressed breast cancer cell proliferation, invasion, and colony formation in vitro and tumor growth in vivo. Furthermore, Rak knockdown was sufficient to transform normal mammary epithelial cells. Therefore, *Rak* acts as a bona fide tumor suppressor gene through the mechanism of regulating PTEN protein stability and function.

## INTRODUCTION

The *PTEN* (phosphatase and tensin homolog deleted from chromosome 10) tumor suppressor gene, located at chromosome 10q23, is frequently mutated in a number of tumor lineages, including glioblastoma, melanoma, and carcinomas of the prostate, breast, and endometrium (Li et al., 1997; Li and Sun, 1997; Steck et al., 1997). PTEN antagonizes the actions of phosphatidylinositol 3-kinase by dephosphorylating the second messenger phosphatidylinositol 3,4,5-trisphosphate (Cantley and Neel, 1999; Di Cristofano and Pandolfi, 2000; Maehama et al., 2001; Wishart and Dixon, 2002), regulating activation of the kinase Akt as well as the downstream cellular survival and growth responses (Bellacosa et al., 1991; Chang et al., 1997; Jimenez et al., 1998; Staal, 1987). PTEN has phosphatase, C2, and PDZ binding domains as well as potential sites of regulation by phosphorylation, including tyrosine phosphorylation, which may contribute to its ability to modulate cell growth and viability.

Emerging evidence shows that complete or partial loss of PTEN protein expression, in addition to gene deletion or mutation, can impact tumor suppression (Salmena et al., 2008). Indeed, PTEN protein levels are reduced in at least 50% of breast cancers, though gene mutations are rare (Hennessy et al., 2005; Brugge et al., 2007; Stemke-Hale et al., 2008). The epigenetic regulation of PTEN expression has been attributed to transcriptional regulation, microRNA, and/or alteration of PTEN protein stability (Salmena et al., 2008). The disruption of PTEN protein stability represents a particularly attractive yet elusive mechanism contributing to its loss in human cancer. Numerous reports have suggested that PTEN stability is regulated by interaction with other proteins and that it is subject to posttranslational modification, particularly phosphorylation (Salmena et al., 2008). To better understand the regulation of PTEN phosphorylation, stability, and function, we used a proteomics-based approach to systematically identify PTEN-binding proteins.

Rak is a 54 kDa tyrosine kinase that belongs to the Src family of kinases (Annerén et al., 2003; Cance et al., 1994; Serfas and

## SIGNIFICANCE

PTEN protein is frequently absent in cancer, particularly breast cancer, despite a lack of gene mutation. The mechanism causing PTEN protein loss has not been defined. Here we demonstrate that the Rak tyrosine kinase phosphorylates PTEN, protecting it from ubiquitin-mediated degradation. Surprisingly, Rak exhibits strong tumor-suppressive activity in vitro and in vivo, at least in part through regulating PTEN protein stability. Thus, *Rak* may function as a tumor suppressor gene, and further understanding of its function may contribute to effective therapeutic approaches for both Rak- and PTEN-defective cancers.

Tyner, 2003). Like all members of the Src kinase family, the Rak kinase possesses an SH domain as well as conserved autoregulatory tyrosine residues in its catalytic domain (Annerén et al., 2003; Cance et al., 1994; Serfas and Tyner, 2003). However, Rak differs significantly from the other Src family members in many structural features, including the presence of a putative bipartite nuclear localization signal and the lack of a consensus myristoylation motif (Annerén et al., 2003; Serfas and Tyner, 2003). In fact, Rak has been shown to be a nuclear protein with growth-inhibitory effects when ectopically expressed in breast cancer cells (Meyer et al., 2003). Notably, the *Rak* gene is located on chromosome 6q21-23, a region that undergoes loss of heterozygosity in 30% of breast cancer cases (Sheng et al., 1996). However, the mechanisms by which Rak and its substrates function in cancer have remained unexplored. In this study, we demonstrate the functional interaction between Rak and PTEN and provide mechanistic evidence that *Rak* functions as a bona fide breast tumor suppressor gene.

## RESULTS

### Rak Is a PTEN-Interacting Protein

To systematically identify proteins involved in regulation of PTEN phosphorylation and/or protein turnover, we carried out immunoprecipitation followed by mass spectrometry. We demonstrated that the Rak tyrosine kinase represents one of the major PTEN-associated proteins (Figure 1A). To validate the mass spectrometry result, we performed immunoprecipitation/western blot analysis and found that PTEN coprecipitated with Rak (Figure 1B, left). Reciprocally, Rak could also be pulled down when an antibody against PTEN was used for immunoprecipitation (Figure 1B, right). This result strongly suggests that endogenous PTEN and Rak physically interact.

To map the binding domain on Rak, we expressed FLAG-tagged wild-type Rak and Rak mutants lacking an SH2 domain (Rak- $\Delta$ SH2) or an SH3 domain (Rak- $\Delta$ SH3) in U2OS cells. We found that PTEN coprecipitated with wild-type Rak and Rak- $\Delta$ SH2, but PTEN binding to Rak- $\Delta$ SH3 was much reduced (Figure 1C). We also created three PTEN mutants, PTEN  $\Delta$ 1–185, PTEN  $\Delta$ 185–352, and PTEN  $\Delta$ 353–403. As shown in Figure 1D, Rak coprecipitated with wild-type PTEN and PTEN  $\Delta$ 353–403, and weakly with PTEN  $\Delta$ 1–185. However, Rak did not bind to PTEN  $\Delta$ 185–352. These results provide evidence that the binding of Rak and PTEN is mediated through the interaction between the SH3 domain of Rak and the C2 domain (and perhaps the phosphatase domain as well) of PTEN.

Previous studies have implicated both Rak and PTEN in breast cancer. We therefore further evaluated the correlation between Rak and PTEN protein expression in breast cancer tissues by linear correlation analysis. Among the 42 cases analyzed, Rak expression showed a strong positive correlation with that of PTEN (32 cases, 76.19%) ( $R^2 = 0.8039$ ,  $p < 0.01$ ) (Figure 1E). High expression of Rak but low expression of PTEN was detected in 7 cases (16.67%), possibly due to *PTEN* deletion or mutation in breast cancer. There were only 3 cases that showed low expression of Rak but high expression of PTEN (Figure 1E). These data suggest a potential link between expression of PTEN and Rak in breast cancer cells.

### Rak Regulates PTEN Protein Stability

Next, we sought to determine whether there is a causal relationship between Rak status and PTEN protein levels. To this end, we established two Rak-overexpressing MCF7 breast cancer cell lines (Rak 44 and Rak 45; Figure 1F, left) and three stable Rak-knockdown nontumorigenic MCF10A cell lines (Rak KD1, Rak KD2, and Rak KD3; Figure 1F, right). Interestingly, we found that Rak positively regulated PTEN protein expression without affecting PTEN mRNA levels (Figure 1G). PTEN protein expression was significantly increased in the Rak-overexpressing MCF7 cells and was abolished in the Rak-knockdown MCF10A cells.

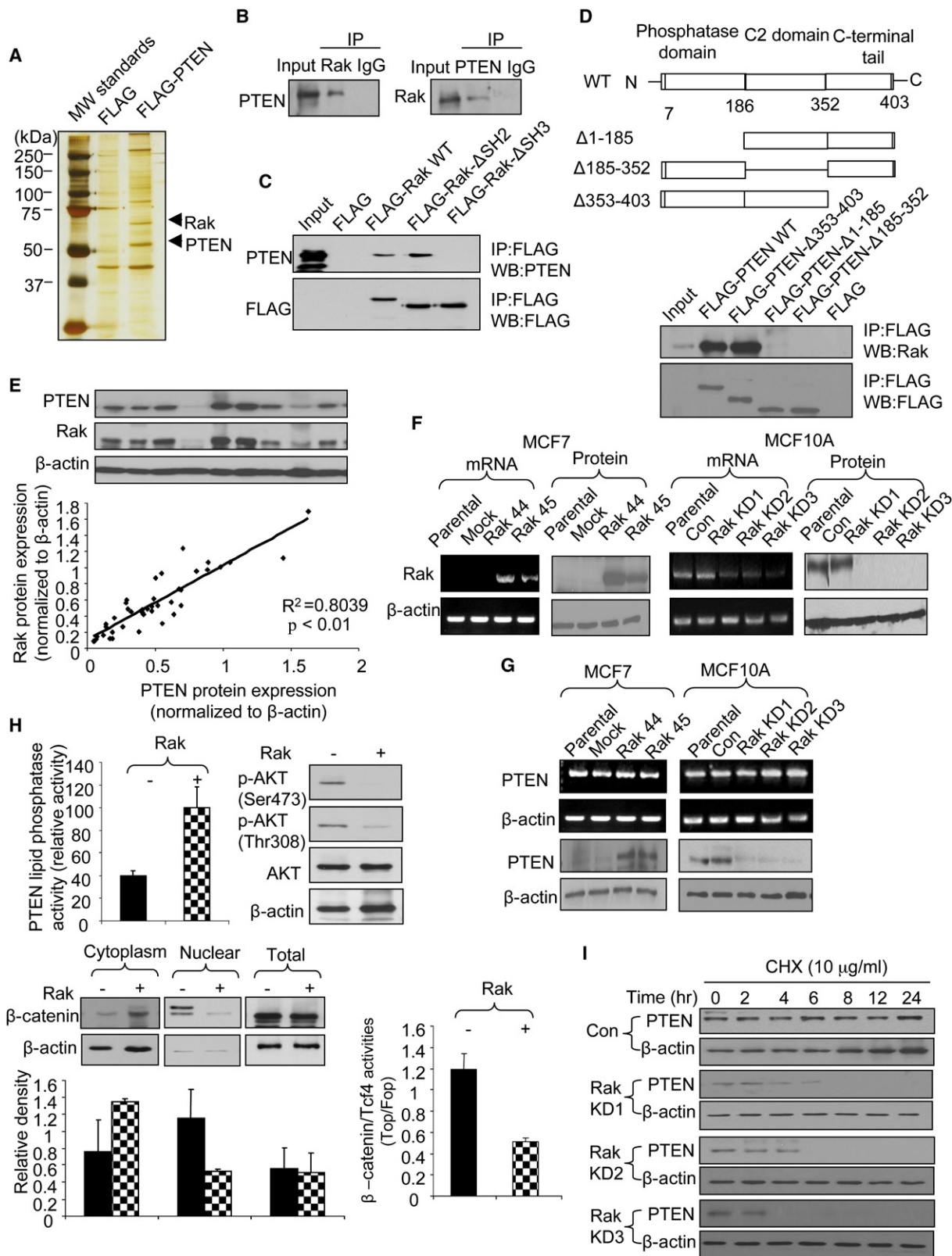
We further tested whether PTEN activity and levels of its downstream targets were also affected by enforced changes in Rak levels. As shown in the top left panel of Figure 1H, the lipid phosphatase activity of PTEN was increased by 60% when Rak was ectopically expressed in MCF7 cells, suggesting that Rak enhances the capacity of PTEN to dephosphorylate phosphatidylinositol. Consistently, Rak overexpression increased the inhibitory effects of PTEN on downstream targets in the PI3K pathway. We found that overexpression of Rak reduced Akt phosphorylation (Figure 1H, top right) and repressed  $\beta$ -catenin nuclear localization and activity as indicated by reporter constructs containing three repeats of wild-type (TOP) or mutant (FOP) Tcf4 (T cell factor)-binding sites (Figure 1H, bottom). PTEN has previously been shown to inhibit nuclear  $\beta$ -catenin accumulation as well as  $\beta$ -catenin transactivation activity (Persad et al., 2001). Thus, these data indicate that enforced Rak expression increases PTEN activity, reducing Akt phosphorylation and blocking its downstream signaling. It is worthwhile to note that the MCF7 cells used in our study express the wild-type Akt and therefore are very suitable for the purpose of this study.

Since Rak binds to PTEN and positively regulates PTEN protein expression, we suspected that Rak might regulate PTEN protein stability. To test this possibility, we compared PTEN protein turnover between control and Rak-knockdown cells in the presence of cycloheximide (CHX), which blocks protein synthesis. As shown in Figure 1I, Rak knockdown led to a reduction in the half-life of PTEN protein from more than 24 hr to less than 6 hr, indicating an essential role of Rak in the stabilization of PTEN protein. Overexpression of Rak also increased the half-life of PTEN in other cell lines, such as the U2OS cells used above (see Figure S1A available online).

### PTEN Ubiquitination Is Accelerated in the Absence of Rak

To determine whether Rak regulates PTEN protein stability through the proteasomal pathway, we treated control and Rak-knockdown cells with a 10  $\mu$ M concentration of the proteasome inhibitor MG132. MG132 treatment substantially increased PTEN protein levels in Rak-knockdown cells but only slightly increased PTEN expression levels in control cells (Figure 2A), indicating that Rak protects PTEN from proteasome-dependent degradation.

It has recently been reported that PTEN protein levels are regulated in part by proteasomal degradation through the ubiquitin ligase NEDD4-1 (Wang et al., 2007). To investigate whether Rak regulates PTEN ubiquitination, we performed an in vivo ubiquitination assay. First, MCF7 cells were cotransfected with



**Figure 1. Rak Binds to PTEN and Regulates PTEN Protein Stability**

(A) Silver staining of the PTEN complex separated by SDS-PAGE. Whole-cell extracts were prepared from U2OS cells transiently transfected with empty vector or FLAG-PTEN.

plasmids encoding FLAG-tagged Rak, His-tagged PTEN, HA-tagged ubiquitin, and/or combinations of control vectors. The expressed His-tagged PTEN was then pulled down from cell lysates with  $\text{Ni}^{2+}$ -nitrilotriacetic acid resin. Subsequently, western blotting against the HA tag was performed to detect ubiquitinated PTEN. As predicted, in the absence of ectopic expression of Rak, PTEN protein was heavily ubiquitinated, with a characteristic ladder indicative of polyubiquitination (Figure 2B, lane 6); in the presence of Rak, PTEN ubiquitination was decreased (Figure 2B, lane 5). Consistently, we also found that PTEN polyubiquitination was increased in the absence of Rak in MCF10A cells and that the treatment of cells with the proteasome inhibitor MG132 caused a robust increase of polyubiquitinated PTEN protein (Figure 2C).

In support of a role for Rak in PTEN polyubiquitination, we demonstrated that Rak altered the binding of PTEN with its E3 ligase NEDD4-1. As shown in Figure 2D, Rak knockdown in MCF10A cells resulted in a substantial increase in the association between endogenous PTEN and NEDD4-1 as indicated by coimmunoprecipitation. Furthermore, PTEN polyubiquitination was significantly decreased in the absence of NEDD4-1 (Figure 2E, lane 2 versus lane 6). Together, our findings strongly indicate that >Rak stabilizes PTEN protein through blocking ubiquitin-mediated proteasomal degradation, likely through reducing PTEN binding to NEDD4-1.

### Rak Phosphorylates PTEN on Tyr336

In light of our finding that PTEN is a binding partner of Rak, we investigated whether PTEN is a Rak substrate. In an *in vitro* kinase assay, Rak could indeed induce PTEN phosphorylation (Figure 3A). Consistently, using a phosphotyrosine antibody, we confirmed the phosphorylation of endogenous PTEN in MCF7 cells stably overexpressing Rak (Figure 3B). Interestingly, when we tested several PTEN mutants in which tyrosine residues that could play a role in the interaction of PTEN with the membrane were mutated, we found that mutation of Tyr336 abolished the phosphorylation of PTEN by Rak. Furthermore, a kinase-dead Rak mutant (K262R) was unable to phosphorylate PTEN (Figure 3C), suggesting that Rak may directly phosphorylate PTEN in intact cells.

### Phosphorylation of Tyr336 by Rak Is Required for Maintenance of PTEN Protein Stability

Our studies indicated that Tyr336 of PTEN is required for PTEN phosphorylation by Rak. To explore the functional significance of this phosphorylation, we ectopically expressed either wild-type or Y336F mutant PTEN in cells and measured protein half-lives. As shown in Figure 3D, Y336F mutant PTEN was present at a moderately lower level than wild-type PTEN. Additionally, when cells were treated with CHX, Y336F mutant PTEN had a half-life of less than 4 hr, compared to greater than 24 hr for wild-type PTEN (Figure 3D). Furthermore, Y336F mutant PTEN levels were markedly increased by treatment with the proteasome inhibitor MG132 (Figure 3E). Consistent with the lack of phosphorylation by Rak, the association between Y336F mutant PTEN and its E3 ligase NEDD4-1 was significantly increased (Figure 3F).

To determine whether phosphorylation of Y336 by Rak is functionally important, we compared the phosphatase activity and the growth-inhibitory effect of ectopically expressed wild-type and Y336F mutant PTEN on *PTEN*-deleted MDA-MB-468 breast cancer cells (mutant PTEN) with or without knockdown of the endogenous *Rak* gene. As shown in the top panel of Figure 3G, we successfully generated two stable Rak-knockdown MDA-MB-468 clones, Rak KD1 and Rak KD3. We then stably expressed either wild-type or Y336F mutant PTEN in the individual Rak KD1 and Rak KD3 MDA-MB-468 clones. As shown in the bottom panel of Figure 3G, the protein level of the Y336F mutant was lower than that of wild-type PTEN in control MDA-MB-468 cells. The lower level of the Y336F mutant was due to its reduced protein stability, which could be rescued by adding the proteasome inhibitor MG132 (Figure S2). Moreover, knockdown of Rak resulted in decreased levels of wild-type PTEN protein without detectable effects on the level of Y336F mutant PTEN, supporting a critical role of Rak in stabilizing PTEN through phosphorylation of Tyr336. Also, as expected, cells expressing Y336F mutant PTEN exhibited significantly lower phosphatase activity compared to cells expressing wild-type PTEN (Figure 3H). Together, these data indicate that phosphorylation of PTEN on Tyr336 by Rak is required for optimal protein stability.

(B) Rak associates with PTEN in cells. Cell lysates from U2OS cells were immunoprecipitated with anti-Rak, anti-PTEN, or preimmune IgG and immunoblotted with anti-PTEN or anti-Rak antibody.

(C) Mapping the PTEN binding domain of Rak. PTEN interacts with the Rak-SH3 domain.

(D) Mapping the Rak binding domain of PTEN.

(E) Correlation of PTEN and Rak protein expression in breast cancer tissue samples ( $R^2 = 0.8093$ ,  $p < 0.01$ ). Protein intensity was normalized to  $\beta$ -actin. The top panel is a representative western blot showing the expression of Rak and PTEN in part of the samples analyzed.

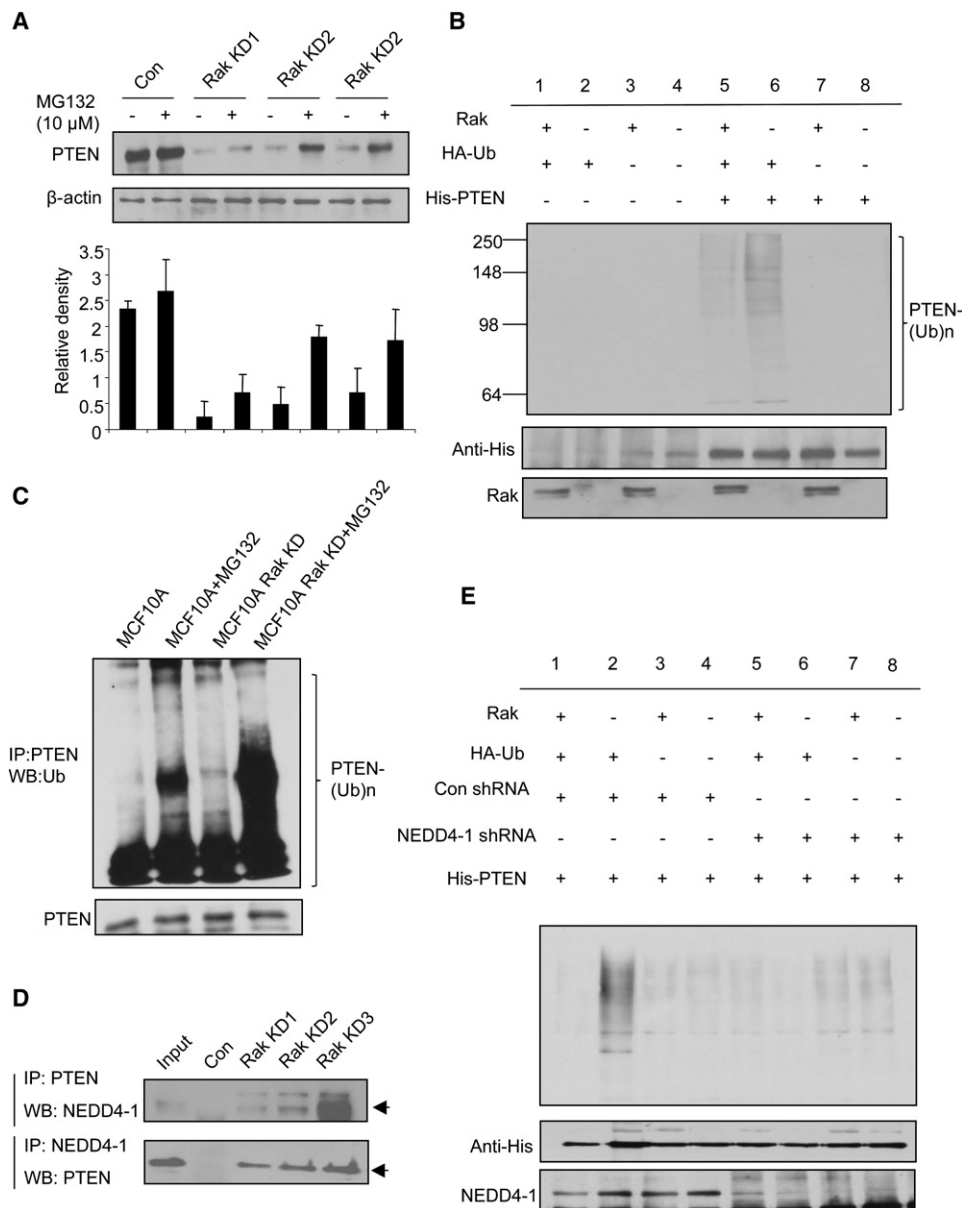
(F) Left: expression of Rak mRNA and protein in control MCF7 cells and Rak-overexpressing MCF7 cells (Rak 44 and Rak 45). Right: expression of Rak mRNA and protein in control MCF10A cells and Rak-knockdown MCF10A cells (Rak KD1, Rak KD2, and Rak KD3). Con, nontarget siRNA included as a negative control.

(G) PTEN is regulated by Rak at a posttranslational level. Left: expression of PTEN mRNA and protein in control MCF7 cells and Rak-overexpressing MCF7 cells. Right: expression of PTEN mRNA and protein in control MCF10A cells and Rak-knockdown MCF10A cells.

(H) Rak enhances PTEN activity and inhibits downstream targets of PTEN. Top left: effect of Rak on PTEN lipid phosphatase activity in Rak-overexpressing MCF7 cells. The capacity of immunoprecipitated PTEN to dephosphorylate water-soluble diC8-phosphatidylinositol 3,4,5-trisphosphate in control or Rak-overexpressing MCF7 cells was evaluated. Top right: Akt phosphorylation at Ser473 and Thr308 is reduced in Rak-overexpressing MCF7 cells compared to MCF7 control cells. Akt protein is shown as a loading control. Bottom left: Rak inhibits nuclear translocation of  $\beta$ -catenin. Lysates from MCF7 control or Rak-overexpressing MCF7 cells were fractionated into nuclear and cytoplasmic fractions, and 40  $\mu\text{g}$  of protein for each fraction was analyzed by western blotting. Bottom right: relative  $\beta$ -catenin/Tcf4 activity in MCF7 control or Rak-overexpressing MCF7 cells measured as the ratio of TOP to FOP luciferase activity. Data represent the mean  $\pm$  SD of at least three independent experiments.

(I) PTEN turnover is reduced in the absence of Rak. Control or Rak-knockdown MCF10A cells were incubated with 10  $\mu\text{g}/\text{ml}$  cycloheximide (CHX) for the indicated periods of time to inhibit protein synthesis. Lysates were harvested from the cells and analyzed by western blotting.





**Figure 2. Ubiquitination of PTEN Is Accelerated in the Absence of Rak**

(A) The proteasome inhibitor MG132 effectively rescues the expression level of PTEN in Rak-knockdown cells. Control (Con) or Rak-knockdown MCF10A cells were treated with 10  $\mu$ M MG132 for 6 hr and probed for PTEN expression by western blotting. Data represent the mean  $\pm$  SD of at least three independent experiments.

(B) Rak inhibits ubiquitination of PTEN. MCF7 cells were cotransfected with plasmids encoding FLAG-Rak, HA-tagged ubiquitin, and His-tagged PTEN. Twenty-four hours after transfection, cells were harvested and lysed. His-tagged PTEN was pulled down with  $\text{Ni}^{2+}$ -nitrilotriacetic acid resin, washed, and subjected to immunoblotting with anti-HA to detect ubiquitinated PTEN.

(C) Endogenous PTEN ubiquitination. Control or Rak-knockdown MCF10A cells were treated with or without 10  $\mu$ M MG132 for 6 hr and then harvested and analyzed. PTEN levels were normalized prior to immunoprecipitation by loading proportionally different amounts of cell extracts.

(D) The interaction between the endogenous E3 ligase NEDD4-1 and substrate PTEN is markedly increased in the absence of Rak. Proteins immunoprecipitated from control or Rak-knockdown MCF10A cell lysates with anti-PTEN or anti-NEDD4-1 antibody were resolved by SDS-PAGE and immunoblotted with anti-NEDD4-1 or anti-PTEN antibody as indicated.

(E) PTEN ubiquitination was significantly decreased by NEDD4-1 knockdown.

In control MDA-MB-468 cells, ectopic expression of wild-type PTEN significantly inhibited cell proliferation. In contrast, the Y336F mutant exerted a less growth-inhibitory effect consistent

with its low level of expression. Strikingly, the growth-inhibitory effect of wild-type PTEN was reversed when Rak was knocked down in the cells (Figure 3I). Together, these results suggest

that phosphorylation of Y336 by Rak prevents PTEN degradation; loss of Rak markedly decreased the ability of PTEN to inhibit tumor cell growth.

### Rak Suppresses Tumorigenicity of Human Breast Cancer Cells

Since we found that Rak regulates the stability of PTEN protein and that mutation of the Rak phosphorylation site on PTEN decreases the growth-inhibitory effect of the *PTEN* tumor suppressor gene, we posited that *Rak* might function as a tumor suppressor gene. To test this possibility, we first examined the phenotypic characteristics of cells ectopically expressing Rak. Interestingly, we observed marked morphologic differences between Rak-overexpressing MCF7 stable cells and control cells. Whereas the control cells were rounded, Rak-overexpressing cells (Rak 44 and Rak 45) developed a more stellate shape closer to the morphology of normal breast cells (Figure 4A). Moreover, overexpression of Rak repressed proliferation (Figure 4B) and growth in soft agar of MCF7 cells (Figure 4C) as well as other cell lines, such as the U2OS cells used above (Figures S1B and S1C). Rak overexpression also suppressed tumor cell invasion in a Matrigel invasion assay that mimics the extracellular matrix in vivo (Figure 4D).

Given that Rak effectively suppressed phenotypes associated with in vitro transformation, we investigated the effect of Rak expression in a xenograft mouse model. Mice were injected in the mammary glands with Rak-overexpressing MCF7 cells or vector control MCF7 cells and monitored weekly for tumor formation. By week 8, all ten of the mice injected with Rak-overexpressing clones remained tumor free, whereas all five of the control mice had developed tumors (Table 1; Figure 4E).

### Rak Knockdown Transforms Normal Mammary Epithelial Cells

As shown above, Rak overexpression suppressed invasion and proliferation of breast cancer cells both in vitro and in vivo. To further assess whether *Rak* is a bona fide breast tumor suppressor gene, we sought to determine whether loss of Rak expression would transform normal mammary epithelial cells.

Transformation activity in vitro and tumorigenicity in vivo were evaluated using the three Rak-knockdown MCF10A cell lines described above. Rak knockdown promoted cell proliferation (Figure 5A), anchorage-independent growth (Figure 5B), and in vitro invasive potential (Figure 5C). Notably, when Rak was depleted in MDA-MB-468 (*PTEN* mutant) cells, its effects on proliferation and anchorage-independent growth were less evident (Figure 3I; Figure S3), suggesting that *PTEN* is one of the major targets mediating Rak suppression of cell transformation.

We also investigated whether Rak knockdown in MCF10A cells would allow tumor formation in xenografts. We injected control cells or individual Rak-knockdown clones into mammary glands of nude mice and monitored tumor growth. Notably, all of the mice injected with Rak-knockdown cells, but none of the mice injected with control vector-expressing cells, developed tumors within 3 weeks after injection (Table 2; Figure 5D). Pathologic analysis of the tumors revealed micropapillary hyperplasia in Rak-knockdown clones (Figure 5E). These results demonstrate that loss of Rak is sufficient to induce tumorigenicity in

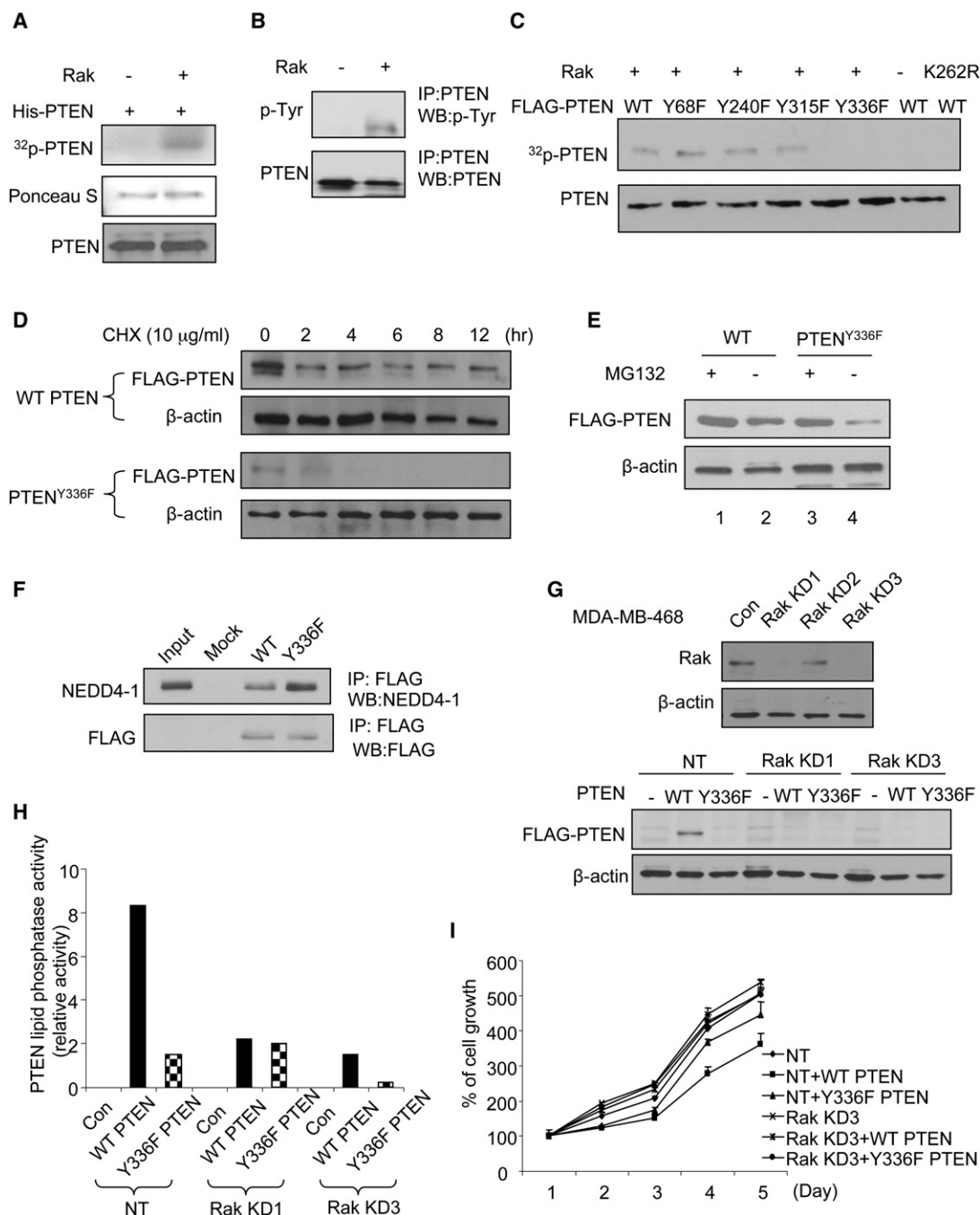
immortalized normal mammary epithelial cells, supporting a role for Rak as a bona fide tumor suppressor in breast cancer.

### DISCUSSION

Previous studies have demonstrated that tumor-associated *PTEN* mutations frequently lead to loss of PTEN protein, with at least 20% of the cases demonstrating high mRNA levels indicative of rapid degradation of mutant PTEN protein in cells (Georgescu et al., 1999). In fact, PTEN tyrosine mutants, with an associated loss of PTEN protein and tumor suppressor activity, have been detected in many types of cancer. However, little is known about the mechanisms that regulate PTEN protein stability and whether the molecules regulating PTEN stability also function as tumor suppressor genes in human cancer. In this study, we found that expression of Rak, a PTEN-interacting protein, strongly correlates with PTEN protein levels in breast cancer tissues, and we demonstrated that Rak positively regulates PTEN protein stability through phosphorylation of PTEN on Tyr336, which in turn prevents PTEN from ubiquitination and degradation.

Recent studies have shown that PTEN protein stability is regulated by ubiquitin-mediated proteasomal degradation through the E3 ligase NEDD4-1. As with PTEN stability, the mechanisms regulating NEDD4-1 and PTEN association have not been fully elucidated. Since Rak enhances PTEN stability, we suspected that phosphorylation of NEDD4-1 or PTEN by Rak might disrupt the interaction between these two molecules and in turn stabilize PTEN protein. In our studies, we found that Rak efficiently phosphorylates PTEN on Tyr336 but has no detectable kinase activity toward NEDD4-1 (data not shown). Notably, Rak depletion increased the interaction between NEDD4-1 and PTEN, suggesting that phosphorylation of PTEN by Rak may inhibit PTEN binding to NEDD4-1 and subsequent PTEN degradation. Indeed, negative regulation of the binding between E3 ligase and substrate as a consequence of tyrosine phosphorylation has previously been reported. For example, Fyn has been shown to phosphorylate the E3 ubiquitin ligase Itch and to reduce its binding to and degradation of its substrate JunB (Yang et al., 2006). Notably, Fyn and Rak belong to the same kinase family, and Itch is a HECT-type E3 ligase, like NEDD4-1. Thus, the regulatory mechanisms are similar, except that Rak phosphorylates the substrate whereas Fyn phosphorylates the E3 ligase. It is tempting to speculate that tyrosine phosphorylation may be a common mechanism for regulation of protein stability, in which tyrosine kinases protect substrates from HECT-mediated degradation. This mechanism is in contrast to the one by which serine/threonine kinases promote F box-containing E3 ligase-mediated ubiquitination and protein degradation.

In addition to elucidating a mechanism that regulates PTEN protein stability, as illustrated in Figure 6, our studies also identified *Rak* as having the potential to act as a tumor suppressor gene in breast cancer. *Rak* is located a region that undergoes loss of heterozygosity in 30% of breast cancer cases. However, the role of *Rak* in breast cancer development is far from clear. Although we identified PTEN as an important substrate for Rak and one that may play crucial role in Rak's tumor suppressor function, we cannot rule out the possibility that Rak may also suppress tumor growth through one or more PTEN-independent



**Figure 3. Rak Phosphorylates PTEN on Tyr336 to Prevent Its Protein Degradation**

(A) Rak induces PTEN phosphorylation. Recombinant Rak was incubated with His-PTEN in the presence of [ $\gamma$ - $^{32}$ P]ATP. PTEN phosphorylation was then examined by autoradiography.

(B) Endogenous PTEN was immunoprecipitated from Rak-overexpressing MCF7 cells or control cells and blotted with antibodies against phosphotyrosine or PTEN. PTEN levels were normalized prior to immunoprecipitation by loading proportionally different amounts of cell extracts.

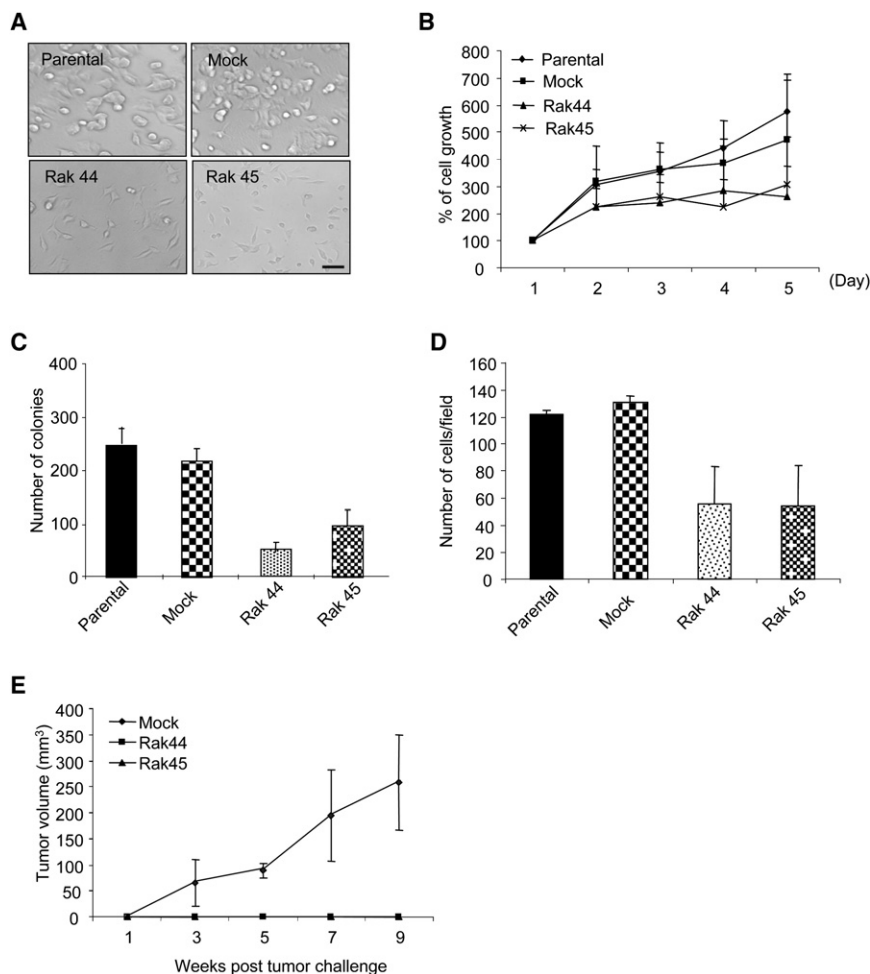
(C) PTEN Tyr336 is phosphorylated by Rak. Recombinant wild-type Rak or its kinase-inactive mutant (K262R) was incubated with FLAG-PTEN or the Y/F PTEN mutants (Y68F, Y240F, Y315F, and Y336F) in the presence of [ $\gamma$ - $^{32}$ P]ATP. PTEN phosphorylation was determined by autoradiography.

(D) Y336F mutant PTEN protein is less stable than wild-type PTEN. Ectopic expression of wild-type (WT) or Y336F mutant PTEN in U2OS cells was measured for 12 hr after CHX treatment.

(E) The proteasome inhibitor MG132 stabilizes Y336F mutant PTEN protein. U2OS cells were treated with 10  $\mu$ M MG132 for 6 hr.

(F) The E3 ligase NEDD4-1 has higher binding affinity to the Y336F mutant than to the wild-type PTEN.

(G) Top: Rak expression levels in nontarget shRNA control (Con) or Rak-knockdown MDA-MB-468 cells were analyzed by western blotting. Bottom: protein expression levels of wild-type or Y336F mutant PTEN were analyzed in control (NT) and Rak-knockdown MDA-MB-468 cell lines.



**Figure 4. Rak Suppresses Tumorigenicity of MCF7 Breast Cancer Cells**

(A) Rak induces a striking morphologic change in monolayer-cultured MCF7 cells. Scale bar = 50  $\mu$ m. (B) Expression of Rak reduces the proliferation of MCF7 cells. Control or Rak-overexpressing MCF7 cells were seeded in a 96-well plate at  $1 \times 10^4$  cells per well. Cell proliferation was measured by MTT assay for 5 days.

(C) Rak suppresses anchorage-independent growth of MCF7 cells. Viable colonies of MCF7 clones in three wells were counted; all soft agar assays were performed in triplicate.

(D) Rak suppresses invasion of MCF7 cells in Matrigel invasion assay. Cells were suspended in collagen type I Matrigel, and the number of invading cells in five fields was counted under 200 $\times$  magnification.

(E) Rak suppresses tumor growth. Cells were injected into the mammary glands of nude mice ( $5 \times 10^6$  cells per mouse), and tumor volumes were measured every 2 days.

Data in (B)–(E) represent the mean  $\pm$  SD of at least three independent experiments per panel.

pathways. PTEN has been reported to play fundamental roles in the maintenance of chromosomal stability as well as stem cell biology. Thus, we speculate that Rak may also participate in these two important biological functions. In fact, our preliminary studies implicate Rak in preventing spontaneous DNA damage (data not shown). Thus, we will determine how Rak regulates genomic integrity and whether Rak is involved in “stemness” of cells in future studies.

## EXPERIMENTAL PROCEDURES

### Cell Culture and Plasmids

U2OS cells and breast cancer cell lines were purchased from the American Type Culture Collection. U2OS cells were maintained in McCoy's 5A medium (Cellgro) supplemented with 10% FBS with glutamine, penicillin, and streptomycin. MDA-MB-468 breast cancer cells were grown in RPMI 1640 medium supplemented with 10% FBS. MCF7 cells were maintained in DMEM/nutrient mixture F12 (Cellgro) supplemented with 10% FBS. MCF10A cells were maintained in mammary epithelial growth medium (Clonetics), a proprietary serum-free medium containing insulin, hydrocortisone, epidermal growth factor, and

bovine pituitary extract. Cells were incubated at 37 $^{\circ}$ C in a humidified incubator with 5% CO<sub>2</sub>.

The pCMV5-3  $\times$  FLAG vector encoding Rak was provided by F.M.-B. The K262R point mutation was created from 3  $\times$  FLAG-Rak using the GeneTailor Site-Directed Mutagenesis System (Invitrogen). The Rak deletion mutants Rak- $\Delta$ SH2 and Rak- $\Delta$ SH3 (Meyer et al., 2003) were provided by R.J.C. pCMV5-3  $\times$  FLAG and pCMV5-3  $\times$  FLAG-PTEN, -PTEN<sup>Y68F</sup>, -PTEN<sup>Y240F</sup>, -PTEN<sup>Y315F</sup>, and -PTEN<sup>Y336F</sup> were provided by G.B.M. The PTEN deletion mutants were generated by PCR (see Figure 1D). His-PTEN (883 pET15b PTEN) was purchased from Addgene. Full-length

NEDD4-1 cDNA was amplified by polymerase chain reaction and subcloned into pGEX-5T-3 (Amersham Pharmacia Biotech). The identity of all plasmids was confirmed by sequencing at the M.D. Anderson Cancer Center DNA Core Sequencing Facility.

### Antibodies and Reagents

Nucleotides 448–1433 were subcloned into pGEX-4T with the sense primer 5'-TTTGGATCCATGAGCAACATcTGTCAGAGG-3' and the antisense primer 5'-TTTCTCGAGTTTCACTGCTACTGGAGTGGT-3', and the protein product was used for immunization for rabbit polyclonal anti-Rak antibody (Proteintech Group, Inc.). Anti-PTEN antibody was purchased from Cell Signaling Technology. Anti-FLAG M2 and anti- $\beta$ -actin were purchased from Sigma. Cycloheximide was obtained from Sigma and used at 10  $\mu$ g/ml (MCF10A cells) or 40  $\mu$ g/ml (U2OS cells); MG132 (carbobenzoxyl-L-leucyl-L-leucyl-L-leucine) was obtained from EMD Biosciences and used at 10  $\mu$ M.

### Affinity Purification of PTEN Protein Complex

U2OS cells were transiently transfected with empty vector or FLAG-PTEN plasmids. Forty-eight hours later, whole cellular extracts were prepared with RIPA buffer (50 mM Tris-HCl [pH 7.4], 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin, 1 mM sodium orthovanadate, and

(H) Lipid phosphatase activity of wild-type or Y336F mutant PTEN was evaluated in the control or Rak knockdown MDA-MB-468 cells.

(I) Wild-type PTEN, but not Y336F mutant PTEN, effectively inhibited the proliferation of MDA-MB-468 cells in a Rak-dependent manner. Data represent the mean  $\pm$  SD of at least three independent experiments.



**Table 1. Tumorigenicity of Orthotopically Implanted Control and Rak-Overexpressing MCF7 Cells**

	Number of Mice with Tumors (%)			
	3 weeks	5 weeks	7 weeks	9 weeks
Control	4/5 (80)	5/5 (100)	5/5 (100)	5/5 (100)
Rak 44	0/5	0/5	0/5	0/5
Rak 45	0/5	0/5	0/5	0/5

$5 \times 10^6$  cells from MCF7 control and two independent Rak-overexpressing MCF7 cell lines (Rak 44 and Rak 45) were injected per mouse into mammary glands of 6-week-old female nude mice. Each cell line was injected in five different mice, and tumor sizes were analyzed.

1 mM NaF) and immunoprecipitated with anti-FLAG M2 affinity gel (Sigma) overnight. Bead-bound immunocomplexes were eluted with  $3 \times$  FLAG peptide and subjected to SDS-PAGE. Silver staining was performed with a SilverSNAP mass spectrometry kit (Pierce). Specific bands were excised and digested, and the peptides were analyzed by mass spectrometry analysis at the M.D. Anderson Cancer Center Proteomics Facility.

#### Preparation of Recombinant Proteins

The plasmids pGEX-5T-3-Rak and -NEDD4-1 were used to express recombinant GST-Rak and NEDD4-1 in *E. coli* strain BL21 (DE3). The GST fusion proteins were expressed by induction overnight at 25°C with 0.1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside in strain BL21 and purified using

glutathione Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The plasmid pET-15b PTEN-His was transformed into strain BL21. The recombinant proteins were expressed and purified by  $\text{Ni}^{2+}$ -NTA affinity chromatography according to standard procedures. The plasmids 3  $\times$  FLAG-Rak, -Rak<sup>K262R</sup>, -PTEN, -PTEN<sup>Y68F</sup>, -PTEN<sup>Y240F</sup>, -PTEN<sup>Y315F</sup>, and -PTEN<sup>Y336F</sup> were transfected into 293T cells, and fusion proteins were purified using a FLAG fusion protein immunoprecipitation kit (Sigma).

#### Reverse Transcriptase-Polymerase Chain Reaction

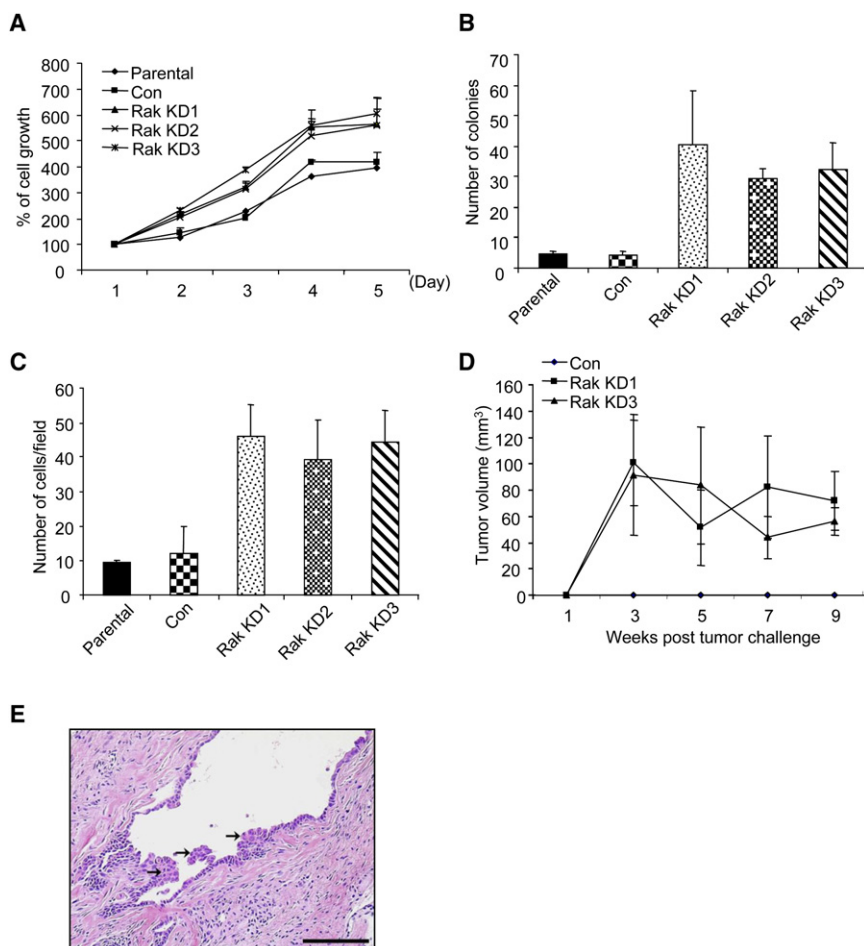
cDNA was transcribed using SuperScript III (Invitrogen) following the manufacturer's instructions. Rak was amplified by polymerase chain reaction using the primers 5'-ATGAGCAACATCTGTCAGA-3' and 5'-TCATCTTATGAAGTTATTGTC-3'. For amplification of PTEN, the primers 5'-GGACGAAGTGGTGAATGATATG-3' and 5'-TCTACTGTTTTGTGAAGTACAGC-3' were used.

#### RNA Interference

Rak knockdown was achieved by RNA interference using a lentiviral vector-based MISSION shRNA (Sigma). Lentiviral particles corresponding to the MISSION shRNA Rak-U22322 target set were used, as well as the MISSION nontarget shRNA control. Specificity and efficacy of the shRNA Rak procedure were controlled by western blotting after transduction and puromycin selection in MCF10A cells. shRNA against NEDD4-1 was purchased from Open Biosystems.

#### Immunoblotting and Immunoprecipitation

Cells were washed in PBS, and cellular proteins were extracted in modified RIPA buffer (50 mM Tris-HCl [pH 7.4], 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin, 1 mM sodium orthovanadate, and

**Figure 5. Loss of Rak Transforms MCF10A Cells and Induces Their Tumorigenicity**

(A) Rak knockdown enhances the proliferation of MCF10A cells. Control or Rak-knockdown cells were seeded in a 96-well plate at  $1 \times 10^4$  cells per well. Cell proliferation was measured by MTT assay for 5 days.

(B) Rak knockdown induces anchorage-independent growth of MCF10A cells. Viable colonies of MCF10A clones in three wells were counted; all soft agar assays were performed in triplicate.

(C) Rak knockdown increases invasion of MCF10A cells in Matrigel invasion assay. Cells were suspended in collagen type I Matrigel, and the number of invading cells in five fields was counted under  $200\times$  magnification.

(D) Rak knockdown induces tumorigenicity of MCF10A cells in nude mice. Control or Rak-knockdown MCF10A cells (Rak KD1 and Rak KD3) were injected into mammary glands of nude mice ( $5 \times 10^6$  cells per mouse), and tumor volumes were measured every 2 days.

(E) Pathologic analysis indicates that micropapillary hyperplasias (arrows) form in nude mice injected with Rak-knockdown MCF10A cells. Scale bar = 100  $\mu$ m.

Data in (A)–(D) represent the mean  $\pm$  SD of at least three independent experiments per panel.

**Table 2. Tumorigenicity of Orthotopically Implanted Control and Rak-Deficient MCF10A Cells**

	Number of Mice with Tumors (%)			
	3 weeks	5 weeks	7 weeks	9 weeks
Control	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
Rak KD1	5/5	4/5 <sup>a</sup>	3/5 <sup>a</sup>	3/5 <sup>a</sup>
Rak KD3	5/5	5/5	5/5	3/5 <sup>a</sup>

$5 \times 10^6$  cells from MCF10A control and two independent Rak-deficient MCF10A cell lines (Rak KD1 and Rak KD3) were injected per mouse into mammary glands of 6-week-old female nude mice. Each cell line was injected in five different animals, and tumor sizes were analyzed.

<sup>a</sup>Some mice had to be sacrificed due to tumor burden starting at 5 weeks after tumor inoculation.

1 mM NaF) for 30 min at 4°C. Lysates were cleared by centrifugation, and proteins were separated by gel electrophoresis. Membranes were blocked in Tris-buffered saline-0.1% Tween 20 (TBS-T)/5% (w/v) milk for 1 hr at room temperature. Membranes were then incubated with primary antibodies diluted in TBS-T/5% (w/v) milk for 2 hr at room temperature. Subsequently, membranes were washed with TBS-T and incubated with horseradish peroxidase secondary antibody (1:4000) (Jackson ImmunoResearch) diluted in TBS-T/5% skim milk. Membranes were washed in TBS-T, and bound antibody was detected by enhanced chemiluminescence (ECL; GE Healthcare).

Immunoprecipitation was performed by incubating lysates from  $5 \times 10^6$  cells with 1  $\mu$ g of antibody at 4°C overnight, followed by addition of 30  $\mu$ l of protein A/G-conjugated agarose beads (Santa Cruz Biotechnology). The precipitates were washed four times with ice-cold PBS, resuspended in 6 $\times$  Laemmli buffer, and resolved by SDS-PAGE followed by immunoblotting.

#### PTEN Lipid Phosphatase Assay

Cells were lysed in 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100 with protease inhibitors. PTEN was immunoprecipitated by rabbit anti-PTEN antibody. The immunoprecipitates were washed and incubated with water-soluble diC8-phosphatidylinositol 3,4,5-trisphosphate (Echelon Research Laboratories) in 100 mM Tris-HCl (pH 8.0), 10 mM DTT at 37°C for 40 min. The supernatant was collected after the reaction and incubated with Biomol Green Reagent (Biomol) at room temperature for 30 min. PTEN activity was measured by colorimetric detection at OD<sub>650</sub> of the release of phosphate.

#### In Vitro Proliferation Assay

Cells were assayed for MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma) reduction, a measure of mitochondrial viability. In brief, cells were harvested from exponential-phase cultures growing in culture medium, counted, and plated in 96-well flat-bottomed microtiter plates (200  $\mu$ l

cell suspensions,  $1 \times 10^4$  cells/ml). Seventy-two hours later, cells were incubated with MTT substrate (20 mg/ml) for 4 hr, the culture medium was removed, and DMSO was added. The optical density was measured spectrophotometrically at 550 nm. Each experiment was repeated at least three times.

#### Soft Agar Assay

Cells were resuspended in DMEM containing 0.5% low-melting agarose (Sigma, type VII) and 10% FBS and seeded onto a coating of 1% low-melting agarose in DMEM containing 10% FBS. Colonies were scored 3 weeks after preparation. Colonies larger than  $\sim 0.1$  mm in diameter were scored as positive.

#### Invasion Assay

Invasion assays were carried out using a cell invasion assay kit (Chemicon) according to the manufacturer's instructions. Briefly, the assay was performed in an invasion chamber consisting of a 24-well tissue culture plate with 12 cell culture inserts. A cell suspension in serum-free culture medium was added to the inserts, and each insert was placed in the lower chamber containing 10% FBS culture medium. After 72 hr incubation in a cell culture incubator, invasiveness was evaluated by staining of cells that had migrated through the extracellular matrix layer and clung to the polycarbonate membrane at the bottom of the insert.

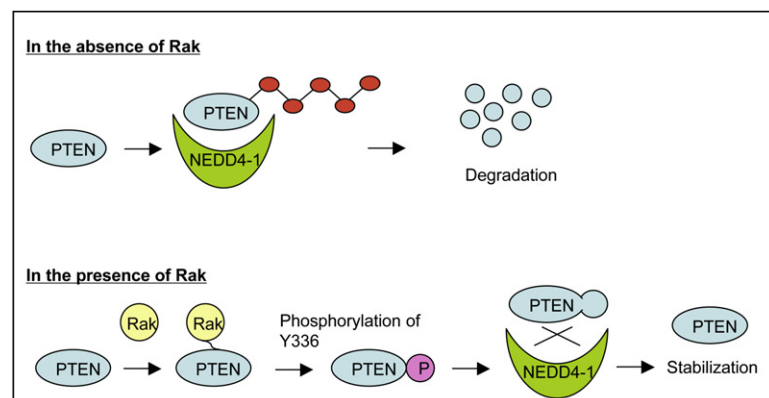
#### Tumor Growth in Nude Mice

Six-week-old female nude mice were used for experiments. All animal studies were conducted in compliance with animal protocols approved by the M.D. Anderson Cancer Center Institutional Animal Care and Use Committee. Before injection of estrogen receptor-positive MCF7 cells (but not MCF10A), mice were implanted subcutaneously with 0.72 mg 17- $\beta$ -estradiol 60-day release pellet (E<sub>2</sub> pellet; Innovative Research of America). Mice were injected in the mammary glands with  $5 \times 10^6$  cells from various cell lines in 100  $\mu$ l PBS. After 1 week, tumors were measured every 2 days. Each cell line was tested in five different animals.

For immunohistochemistry, tumor tissue samples were fixed in 10% buffered formalin and processed for histopathologic evaluation by paraffin embedding and hematoxylin and eosin staining.

#### Transfection and In Vivo Ubiquitination Assay

Cell culture transfection was performed using Lipofectamine 2000 (Invitrogen). For in vivo PTEN ubiquitination assay, transfection was performed when MCF7 cells in 10 cm plates reached  $\sim 90\%$  confluence. Plasmids encoding PTEN, Rak, and HA-tagged ubiquitin were used in transfections in individual experiments. Forty-eight hours after transfection, cells were harvested, washed with PBS, pelleted, and lysed in RIPA buffer. The cell lysates were then subjected to affinity purification with Ni<sup>2+</sup> resin (Amersham Pharmacia Biotech) to precipitate His-tagged PTEN with the resin. These precipitated PTEN proteins were separated by SDS-PAGE and detected by immunoblotting with anti-HA antibody.



**Figure 6. Schematic Model of How Rak Phosphorylates PTEN and Protects PTEN from Ubiquitin-Mediated Degradation**

Rak phosphorylates PTEN on Tyr336, which reduces PTEN binding to the E3 ligase NEDD4-1 and subsequent PTEN degradation.

### In Vitro Kinase Assay

Kinase reactions were performed by mixing purified His-PTEN with or without purified Rak in kinase assay buffer containing 10  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP, 10 mM Tris-HCl (pH 7.4), 10 mM MgCl<sub>2</sub>, 1.2 mM MnCl<sub>2</sub>, 2 mM sodium orthovanadate, and 20  $\mu$ M ATP for 20 min at 30°C. Reactions were terminated by adding SDS-PAGE sample buffer and boiling at 100°C for 5 min. Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes for exposure to X-ray film.

### Analysis of PTEN and Rak Expression in Breast Cancer Samples

The proteins in 42 breast cancer tissue samples obtained from the institutional breast cancer bank were extracted by RIPA buffer and analyzed for expression of Rak and PTEN. After western blotting analysis, the intensity of Rak and PTEN bands was quantitated, and the relationships between Rak and PTEN protein expression were analyzed by linear regression.  $p < 0.05$  was considered significant. This study was performed according to protocols approved by the M.D. Anderson Cancer Center Institutional Review Board, and all subjects provided written informed consent.

### SUPPLEMENTAL DATA

The Supplemental Data include three figures and can be found with this article online at [http://www.cancercell.org/supplemental/S1535-6108\(09\)00043-9](http://www.cancercell.org/supplemental/S1535-6108(09)00043-9).

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